N-terminal sequence determination and secondary structure analysis of extrinsic membrane proteins in the water-splitting complex of spinach

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The N-terminal sequences of the 16 and 23 kDa extrinsic membrane proteins in photosystem II of spinach have been determined by solid-phase sequencing. The sequence of the 23 kDa protein has been corroborated by a small 3 kDa CNBr fragment confirming partial N-terminal processing by a protease of unknown nature. A secondary structure analysis of the 23 kDa protein by circular dichroism has been performed indicating a β-structure content of 61% with only 14% α-helix conformation.

Photosystem II *CD* *Photosynthesis* *Water splitting* *Membrane protein* *N-terminal sequence*

1. INTRODUCTION

In photosystem II of algae and higher plants 3 extrinsic proteins of 16, 23 and 33 kDa have been detected which are localized at the inner surface of the thylakoid membrane [1–4]. These proteins obviously express regulatory functions in the water-splitting system. From the experimental material so far available it is evident that the 33 kDa protein is involved in binding and/or stabilization of the manganese cluster which is considered to play an essential role in the photosynthetic water-splitting process [4–7]. The other two proteins are also required for optimal activation of oxygen evolution. They function as modulators of the action of certain inorganic cofactors, such as Ca^{2+} and Cl^- which are involved in the water-splitting process [4,7–12]. One of the prerequisites for the elucidation of the mechanism of action of these proteins is the analysis of their molecular structure. Here, we report on the N-terminal amino acid sequences of the 16 and 23 kDa proteins and on the secondary structure of the latter component.

2. MATERIALS AND METHODS

CNBr and acetonitrile (Lichrosolv grade) were products of Merck (Darmstadt). Trypsin was purchased from Boehringer (Mannheim). Aminopropyl glass (APG) was prepared by reaction of controlled pore glass CPG 10/75, 200–400 mesh (Serva, Heidelberg) with 3-aminoethyltriethoxysilane (Pierce, distributed in FRG from Karl OHG, Geisenheim). 4-*N,N*-Dimethylaminoazobenzene 4′-isothiocyanate (DABITC) was purchased from Pierce or Fluka (Neu-Ulm). *p*-Phenylenediisothiocyanate was obtained from Fluka. Column material was AcA 54 from LKB.

2.1. *Digestion of the 23 kDa protein with CNBr*

1–3 mg of the freeze-dried 23 kDa protein were dissolved in 1 ml of 70% trifluoroacetic acid and digested with 1 g CNBr for 24 h at room temperature. The excess of CNBr was removed in vacuo. The residue was dissolved in 2 ml of 0.1% trifluoroacetic acid (pH 2.2). The CNBr fragments

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were separated by reversed-phase HPLC on a Pharmacia Pro RPC HR 5/10 column.

2.2. *Sequence determinations*

Protein (~2–4 nmol) and peptide fragments were coupled to aminopropyl glass via *p*-phenylenediisothiocyanate according to Wachter et al. [13]. CNBr peptides were also coupled by the homoserine lactone method. Automated solid-phase Edman degradations were performed with 4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate/phenyl isothiocyanate as described by Salnikow et al. [14]. In the first degradation step phenyl isothiocyanate was used exclusively for most samples in order to minimize background interference in the subsequent cycles; thus the amino-terminal amino acid was not identified. Analyses of the 4-*N,N*-dimethylaminoazobenzene 4′-thiohydantoins were performed by two-dimensional micro thin-layer chromatography on polyamide [15] and, in addition, by high-performance liquid chromatography (HPLC) on reversed phase with a methanol gradient [16]. The thiohydantoins of Leu and Ile were differentiated using an isocratic HPLC system with aqueous acetonitrile [17].

A Pharmacia FPLC system was used for protein purification. Circular dichroism (CD) spectra were measured with a Jasco J-20 spectropolarimeter.

3. RESULTS AND DISCUSSION

3.1. *Protein purification*

The extrinsic membrane proteins of the water-splitting complex were purified either as described in [18] or using the following procedure: Photosystem II peptides were prepared from spinach thylakoid membranes by treatment with Triton X-100 [19,20]. The 16 and 23 kDa proteins were extracted from these particles with 0.5 and 1 M NaCl solutions. The 33 kDa protein was the main component of the 1 M $CaCl_2$ extract. The extracts were centrifuged in a Beckman Ti-60 rotor at approx. 200000 × *g* for 5 h to remove pigment containing material of high M_r, desalted by dialysis and freeze-dried. The lyophilisates were taken up in a minimum volume of either 20 mM Tris or 20 mM NH_4HCO_3 buffer (pH 8.0). The 33 kDa protein was obtained in pure form by AcA 54 gel filtration from the lyophilisate of the $CaCl_2$ extract, as demonstrated in fig.1a,b. The 16 and 23 kDa proteins appear only partially separated under these conditions starting from the 0.5 or 1 M NaCl extract (fig.1b). Therefore, they were first separated by an FPLC technique using anion-

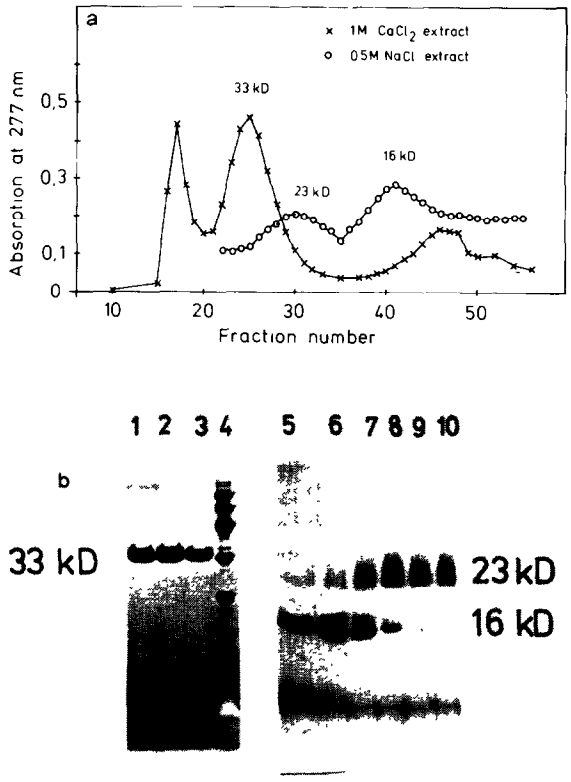

Fig.1. (a) Purification of the extrinsic membrane proteins in the water-splitting complex of spinach by AcA 54 gel filtration. A 0.5 M NaCl and a 1 M $CaCl_2$ extract of photosystem II particles were desalted and freeze-dried. The lyophilisates were dissolved in a minimum volume of either 20 mM Tris or 20 mM NH_4HCO_3 buffer (pH 8.0) and loaded on the column (36 × 2.5 cm). The proteins were eluted with the same buffers. Flow rate, 50 ml/h; temperature, 4°C. (b) SDS-15% polyacrylamide gels of fractions obtained from the AcA 54 gel filtration experiments shown in a. Lanes: 1–3, aliquots of fractions 23, 26 and 28 of gel filtration of the 33 kDa protein (1 M $CaCl_2$ extract); 5–10, aliquots of fractions 31, 33, 35, 37, 41 and 44 of gel filtration of the 16 and 23 kDa proteins (0.5 M NaCl extract); 4, marker proteins – phosphorylase (94 kDa); bovine serum albumin (67 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

exchange chromatography on Pharmacia Mono Q HR 5/5 [21]. The 23 kDa protein was eluted with a linear gradient ranging from 0 to 0.3 M NaCl either in 20 mM Tris or 20 mM NH_4HCO_3 buffer at pH 8.0. The 16 kDa protein was excluded from the Mono Q column under these conditions. Both proteins were finally purified by AcA 54 gel filtration. Using this procedure, highly purified, desalted protein material is obtained which is needed for protein chemical and reconstitution experiments.

3.2. *N-terminal sequencing of the 16 and 23 kDa proteins*

The N-terminal sequences of the 16 and 23 kDa proteins have been determined by solid-phase sequencing, as compiled in table 1. The N-terminal sequence of the 16 kDa protein shows an unusual proline-rich stretch of amino acids with obviously hydrophobic character (residues 5–14) suggesting a distinct conformation for this part of the protein. The N-terminal sequence of the 23 kDa protein has been obtained for 21 residues. Positions 11, 13 and 14 could not be identified in the course of the N-terminal sequencing of the whole protein coupled to the aminopropyl glass via amino groups. However, these residues could be assigned as lysines by sequencing of the small N-terminal CNBr fragment (approx. 3 kDa) which had been attached to the matrix by the homoserine lactone method.

Since the 23 kDa protein possesses only 1 Met residue [18], a second larger CNBr fragment of approx. 20 kDa is to be expected. This has been isolated, however, in impure form – essentially contaminated with uncleaved 23 kDa protein – precluding unambiguous sequencing at present.

Apart from the presented main N-terminal sequence for some preparations of the 23 kDa protein a more or less pronounced side sequence starting with residue 10 (Gly) has been observed. This phenomenon has also been corroborated by the sequence of the 3 kDa CNBr fragment which during degradation proved to consist of two peptides implying partial cleavage of the ^{9}Phe-^{10}Gly main chain. Whether this partial N-terminal processing of the 23 kDa protein is due to thylakoid-specific proteases solubilized by Triton X-100 or is induced by other unspecific cytosolic enzymes is not known at present. Incidentally, partial N-terminal processing has also been observed for the large subunit of ribulosebisphosphate carboxylase/oxygenase from tobacco [22].

Table 1

Partial sequences of the extrinsic membrane proteins in photosystem II of spinach

16 kDa protein

N-terminal sequence:

```
1                5                  10                  15                  20
X-Ala-Arg-Pro-Ile-Val-Val-Gly-Pro-Pro-Pro-Pro-Leu-Ser-Gly-Gly-Leu-Pro-Gly-Thr--Glu-Asn-Ser-Asp-
25
Gln-Ala-X-X-Gly....
```

23 kDa protein

N-terminal sequence:

```
1                5                  10                  15                  20
X-Tyr-Gly-Glu-Ala-Ala-Asn-Val-Phe-Gly--X-Pro-X-X-Asn-Thr-Glu-Phe-Thr-Pro-Tyr....
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small CNBr fragment:

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1                5                  10                  15
X-Tyr-Gly-Glu-Ala-Ala-Asn-Val-Phe-Gly-Lys-Pro-Lys-Lys-Asn-Thr-Glu-Phe....
                                   Gly-Lys-Pro-Lys-Lys-Asn-Thr-Glu-Phe-Thr....
```

1

X, the amino terminus could not be identified, because the amino group is coupled to the aminopropyl glass matrix; X, not identified (may be Lys or Cys for internal residues); Ile/Leu, Ile and Leu have not been differentiated

3.3. *A secondary structure analysis of the 23 kDa protein on the basis of its CD*

The CD spectrum of the 23 kDa protein was measured between 195 and 240 nm in the region of the peptide backbone chromophores, as shown in fig.2. The protein was dissolved in 20 mM NH_4HCO_3 buffer (pH 8.0) containing 0.1 M NaCl. A secondary structure analysis was performed using a method introduced by Provencher and Glöckner [23]. In this procedure the CD spectrum of the 23 kDa protein was analyzed as a linear combination of the CD spectra of 16 proteins whose secondary structure are known from X-ray crystallography. The following secondary structure data have been obtained: α-helix, 14%; β-structure, 61%; remainder, 25%. Obviously the 23 kDa protein is rich in β-structure, while α-helical segments are less representative structural elements of this polypeptide.

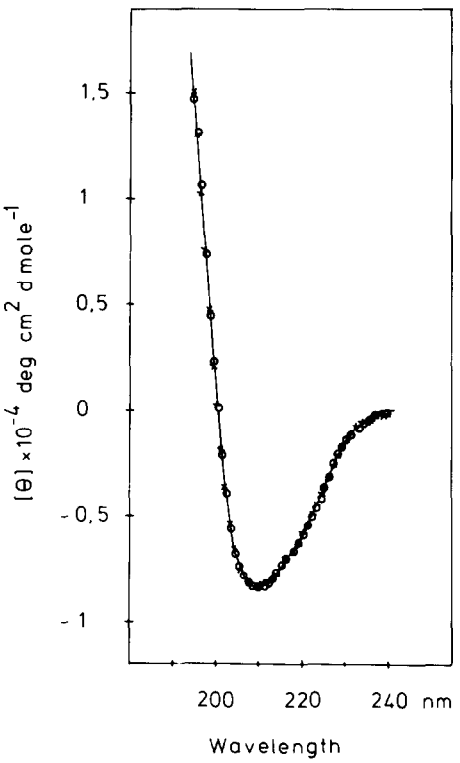

Fig.2. CD spectrum of the 23 kDa protein in the range of 195 and 240 nm. The protein was dissolved in 20 mM NH_4HCO_3 buffer (pH 8.0) containing 0.1 M NaCl. A secondary structure analysis was performed using the method of Provencher and Glöckner [23]. Experimental data (×); fit to data (○). Optical path length, 1 mm; temperature, 25°C.

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